

MycAssay™ Pneumocystis

Applied Biosystems 7500

REF 080-075

Intended Use:

MycAssay™ Pneumocystis is a Real-Time PCR kit for the detection of *Pneumocystis jirovecii* genomic DNA using the Applied BioSystems 7500 in combination with SDS software version 1.4.

Principles of the Assay

Following mixing of the reagents in the MycAssay™ Pneumocystis kit with a sample containing *Pneumocystis* target DNA sequence, (a portion of the *Pneumocystis* mitochondrial ribosomal large sub-unit), thermocycling will result in DNA amplification occurring. The assay also contains an Internal Amplification Control (IAC) sequence, a DNA fragment not present in *Pneumocystis*, other fungal, bacterial or human genomes, to detect PCR inhibitory substances and confirm the functionality of the assay reagents.

The amplified DNA targets are detected with Molecular Beacons, single-stranded oligonucleotide hybridization probes that form a stem-and-loop structure. The loop contains a probe sequence that is complementary to a target sequence, and the stem is formed by the annealing of complementary arm sequences that are located on either side of the probe sequence. A fluorophore, which fluoresces when excited by light of the appropriate wavelength, is covalently linked to the end of one arm and a quencher, which suppresses the fluorescence of the fluorophore when in close physical proximity, is covalently linked to the end of the other arm. Molecular Beacons do not fluoresce when they are free in solution. However, when they hybridise to a nucleic acid strand containing a target sequence they undergo a conformational change that enables them to fluoresce. The amount of fluorescence at any given cycle, or following cycling, depends on the amount of specific amplicons present at that time. The SmartCycler® Real-Time PCR System simultaneously monitors the fluorescence emitted by each beacon.

Precautions

- The kit is for Research Use Only. It is Not for Use in Diagnostic Procedures.
- The kit is intended for use only by laboratory professionals. Procedures are required for non-aerosol manipulations of specimens. Standard precautions and institutional guidelines should be followed in handling all samples. A Material Safety Data Sheet is available from Myconostica Ltd.
- This test is only for use with the Applied BioSystems 7500 with SDS software version 1.4.
- Do not use reagents or controls if the protective pouches are open or broken upon arrival.
- Reagents and controls are not interchangeable between kits with differing lot numbers.
- Never pool reagents or controls from different tubes even if they are from the same lot.
- Never use the reagents or controls after their expiry date.
- Reagents and controls should not be refrozen or reused after opening.
- Wear protective clothing and disposable gloves while handling kit reagents.
- Avoid microbial and deoxyribonuclease (DNase) contamination of reagents when removing aliquots from tubes.
- The use of sterile DNase-free, low-retention disposable filter-tipped or positive displacement pipette tips is recommended.
- Use a new tip for each specimen or reagent.
- Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.
- To avoid contamination with *Pneumocystis* or IAC amplicons, do not open the reaction tubes post-amplification.
- Do not eat, drink or smoke in areas where specimens or kit reagents are being handled.
- Low concentrations of DNA can be unstable if not stored correctly. It is recommended that DNA extractions are stored at -80°C to preserve their integrity. Multiple rounds of thawing and refreezing should also be avoided whenever possible.

Kit Contents

Description

The kit consists of five 3-compartment sealed foil pouches, each of which can be used separately. Each pouch contains sufficient reagents for 8 reactions.

		<u>Volume</u>
Tube 1 (Orange Cap)	dNTPs MgCl ₂ Buffered solution of DNA Polymerase complex	66 µL
Tube 2 (Blue Cap)	<0.01% Primers <0.01% Molecular Beacons <0.0001% Internal Amplification Control (IAC) The IAC is a recombinant DNA plasmid harbouring a non-infective sequence unrelated to either target (<i>Pneumocystis</i>) sequence Tris-HCl Buffer	66 µL
Tube 3 (Clear Cap)	Negative Control Water	25 µL
Tube 4 (Black Cap)	Positive Control <0.0001% Positive Control DNA The Positive Control molecule is a recombinant plasmid harbouring the <i>Pneumocystis</i> target sequences Tris-HCl Buffer	25 µL

The kit also contains:

- MycAssay™ Pneumocystis Myconostica Protocol CD-ROM
- Instructions for Use
- Certificate of Analysis

Storage

The kit should be stored frozen (-15 to -25 °C) until the expiry date indicated on the kit box label, at which time it should be disposed of according to local regulations.

Once a pouch has been opened, the contents must be used immediately, not re-frozen or re-used.

Equipment/Materials required and not provided

- Applied BioSystems 7500 Real-Time PCR System (including user manual, attached computer and SDS software version 1.4).
- MicroAmp® Optical 8-tube strip (Applied BioSystems, part number:4316567).
- MicroAmp® Optical 8-cap strip (Applied BioSystems, part number:4323032).
- Micro centrifuge with 0.2 mL PCR tube adapter.
- Vortex mixer
- Support rack for PCR tubes.
- Micropipettes (volumes required 7.5 µL – 20 µL)
- Sterile low-retention filtertips
- Disposable gloves, powderless
- Proprietary DNA decontaminating solution
- Permanent marker pen
- DNA isolation kit (see below)

Sample

The sample for the MycAssay™ Pneumocystis assay is *Pneumocystis* genomic DNA.

Procedural Notes

- Read the entire protocol before commencing
- The entire MycAssay™ Pneumocystis process (excluding DNA extraction) takes approximately 2 hours, dependent on the number of samples tested.
- Setting up of the test should be performed in a PCR workstation or pre-PCR laboratory. If a PCR workstation is not available, then the test should be set-up in a dedicated area of the laboratory¹, which is regularly cleaned with DNA decontaminating reagents.
- However, avoid using DNA decontaminating reagents during the Real-Time PCR set-up as they can inhibit the assay.
- Use micropipettes for the transfer of fluids. Dedicated micropipettes should be used for the set-up of these reactions and they should be regularly decontaminated.
- Low-retention filter-tips are recommended for use to ensure that no DNA is lost during the set-up procedure.
- **Exercise caution when handling Tube 4. This contains template DNA material and contamination could result in false positive test results.**
- Wear gloves at all times.
- All tubes must be capped following use and prior to disposal.

¹ For example see Mifflin, T. E. (2003). Setting up a PCR Laboratory. *In* PCR Primer, 2nd Ed. (eds. Dieffenbach and Dveksler). Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY. USA.

- Accurately note the positions of samples when multiple samples are being processed.

Procedure for Use:

1. Real-Time PCR Set-Up

- 1.1 To begin, switch on the AB7500 Real-Time PCR System (instrument and associated computer) and launch the SDS v1.4 software. Enter usernames and passwords if required.
- 1.2 Ensure the work area has been cleaned using DNA decontaminating reagents and allowed to dry completely; avoid use during assay set-up as excess cleaning solution may inhibit the PCR reactions.
- 1.3 A pouch contains one each of Tube 1, Tube 2, Tube 3 and Tube 4. There are sufficient reagents in one pouch to run 8 reactions. At least one positive control and one negative control reaction must be performed per run where the reagents are from a single kit lot. One pouch therefore can analyse 6 test samples. If more than 6 samples need to be tested, more than one pouch can be used if the pouches used are from the same kit lot. A maximum of 38 test samples may be tested using the 5 pouches in a kit.
- 1.4 Calculate the number of reactions required, referring to the table below:

Number of Pouches	Maximum number of test samples
1	6
2	14
3	22
4	30
5	38

- 1.5 Remove the appropriate number of pouches from the freezer. Do not use any pouch that is no longer sealed. If the test samples were frozen after extraction, also remove these from the freezer.
- 1.6 Tear open the required number of pouches and remove the tubes. If more than one pouch is being used, but only one set of positive and negative controls are being run, it is only necessary to remove Tubes 3 and 4 from one pouch. **Exercise caution when handling Tube 4. This contains positive**

control DNA material and contamination could cause false positive test results.

- 1.7 Allow the tubes' contents to thaw by placing on the laboratory bench for 5-10 minutes, ensuring that the contents of each tube are completely thawed before proceeding. Vortex to mix the tubes' contents and the test samples; follow by a short spin in a microcentrifuge to ensure collection of all the contents at the base of the tubes before use.
- 1.8 Place the required number of PCR tubes in the support rack. **Never touch the bottom of the reaction tubes with your hands.**
- 1.9 Always set up the negative control first, followed by the test samples. The positive control should always be set up last.
- 1.10 Reagent and DNA volumes are shown in the table below:

Reagent	Reaction		
	Negative control	Test sample	Positive control
Tube 1 (Orange cap)	7.5 µL	7.5 µL	7.5 µL
Tube 2 (Blue cap)	7.5 µL	7.5 µL	7.5 µL
Tube 3 (Clear cap)	10 µL	-	-
Test Sample	-	10 µL	-
Tube 4 (Black cap)	-	-	10 µL
Total volume	25 µL	25 µL	25 µL

- 1.11 Add reagents in the order shown in the table above; Tube 1, then Tube 2, followed by the template (Negative control, Test sample, or Positive control). Take care when taking aliquots from Tube 1; the liquid is slightly viscous and can stick on the inner ridge of the tube. If this happens, re-spin to collect the final contents in the base of the tube before attempting to remove the final aliquots.
- 1.12 Use a new pipette tip for every liquid transfer. Re-cap each reagent tube after use and immediately discard it, and any remaining contents, into a sealable clinical waste container. Unused reagents cannot be saved for later use.
- 1.13 Take extra care when pipetting Tube 4 (positive control DNA) to ensure it does not contaminate any other reaction tube. Closing the lids on the other

- reaction tubes before opening Tube 4 can reduce the risk of cross-contamination.
- 1.14 Make sure all reaction tube caps are firmly closed. Make a note of the positions of each sample in the strip tubes. Label (for example: on the lid) the first tube of each strip if more than one strip tubes are used. Spin down the reaction tubes for 10 seconds using a mini centrifuge with 0.2 mL PCR tube adapter. Visually check that there are no bubbles present in the reaction mixtures.
- 1.15 Proceed to Section 2 promptly. MycAssay™ Pneumocystis reactions are stable on the bench for up to 60 minutes.
- 1.16 Following the PCR set-up ensure the work area is thoroughly cleaned using DNA decontaminating reagents.

2. Performing the run

- 2.1 Open up the AB 7500 SDS software version 1.4 and enter your username and password.
- 2.2 Insert the **MycAssay Pneumocystis Myconostica Protocol CD-ROM**.
- 2.3 In the **Quick Startup** menu, select the first option; **Create New Document...**
- 2.4 Choose the settings as shown below. Select the template **MycAssay Pneumocystis SDS1_4v3_1.sdt** from the CD-ROM via **Browse...**
- 2.5 Give the run an appropriate Plate Name. An example is shown below:

The screenshot shows a software window titled "New Document Wizard" with a close button (X) in the top right corner. The window has a blue header bar. Below the header, the title "Define Document" is followed by the instruction: "Select the assay, container, and template for the document, and enter the operator name and comments." The main area of the window contains several input fields and buttons. The "Assay:" field is a dropdown menu with "Standard Curve (Absolute Quantitation)" selected. The "Container:" field is a dropdown menu with "96-Well Clear" selected. The "Template:" field is a dropdown menu with "MycAssay Pneumocystis SDS1_4v3_1.sdt" selected, and a "Browse..." button is to its right. The "Run Mode:" field is a dropdown menu with "Standard 7500" selected. The "Operator:" field is a text box containing "your name". The "Comments:" field is a large text area with a vertical scrollbar. The "Plate Name:" field is a text box containing "Pneumocystis_Z3MAR10_NAME". At the bottom of the window are four buttons: "< Back", "Next >", "Finish", and "Cancel".

New Document Wizard

Define Document
Select the assay, container, and template for the document, and enter the operator name and comments.

Assay: Standard Curve (Absolute Quantitation)

Container: 96-Well Clear

Template: MycAssay Pneumocystis SDS1_4v3_1.sdt

Run Mode: Standard 7500

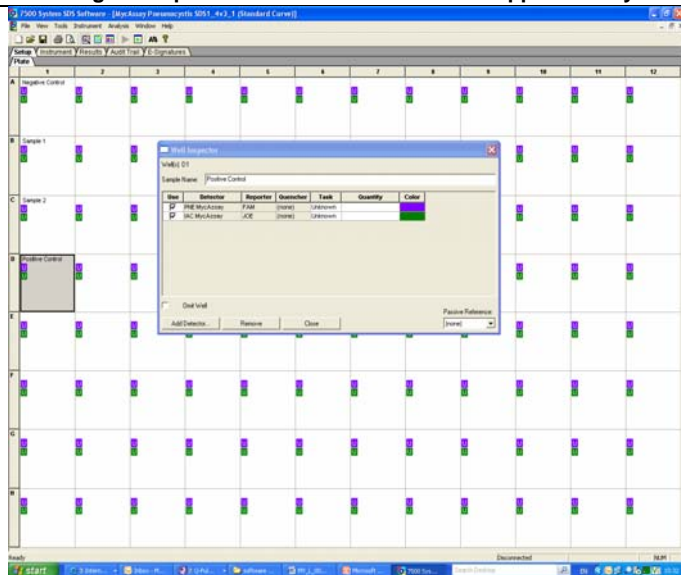
Operator: your name

Comments:

Plate Name: Pneumocystis_Z3MAR10_NAME

< Back Next > Finish Cancel

- 2.6 Click **Finish**. A new document will open containing the PCR parameters and detectors automatically set for this assay. In the **Plate** view of the **Setup** tab, use **Well Inspector** (select a well and press Ctrl+1 or right-click with the mouse) to name the wells according to the positions of the samples in 1.14. For example:



- 2.7 When all the wells are named appropriately, save the run, keeping the **Plate Name** as the file name
- 2.8 Start the run in the **Instrument** tab by clicking on the **Start** button.
To determine how long the run will take to complete, a countdown is shown next to the **Start** button.

3. Data Analysis and Interpretation

- 3.1 Once the run has finished, click on the green arrow on the top menu bar to update.
- 3.2 Open the **Amplification Plot** view of the **Result** tab. On the right hand side set the thresholds for each channel as follows (The thresholds are for use with MicroAmp® Optical PCR strip tubes only and may vary if different plastic consumables are used).

PNE MycAssay = 10000

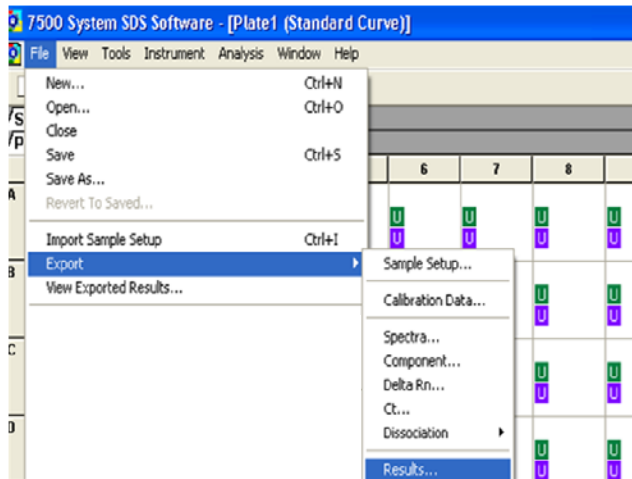
IAC MycAssay = 4000

- 3.3 The **Manual Baseline** should remain at 3 - 15 for both detectors. Click the **Analyze** button to activate these changes. For example:

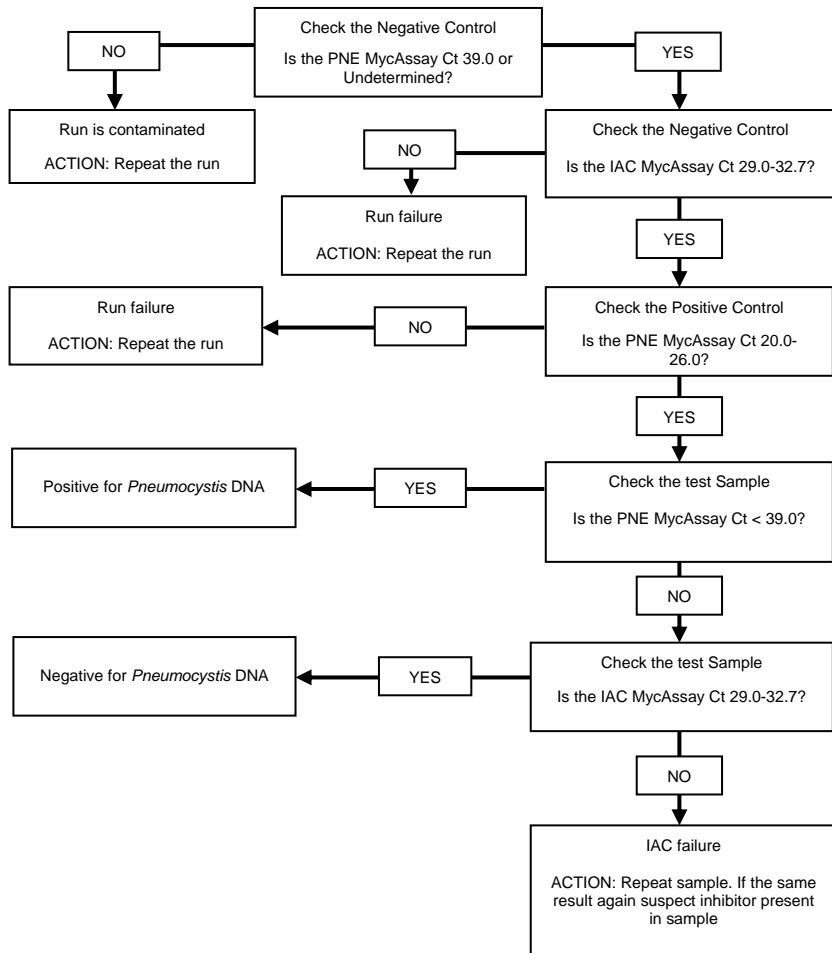
The screenshot displays two side-by-side configuration panels for the IAC MycAssay and PNE MycAssay detectors. Each panel includes dropdown menus for 'Data' (set to 'Delta Rn vs Cycle') and 'Detector' (set to 'IAC MycAssay' and 'PNE MycAssay' respectively), and a 'Line Color' dropdown set to 'Detector Color'. Below these are 'Analysis Settings' sections. For the IAC MycAssay panel, 'Manual Ct' is selected with a threshold of 4.0e+003, and 'Manual Baseline' is selected with a start cycle of 3 and an end cycle of 15. For the PNE MycAssay panel, 'Manual Ct' is selected with a threshold of 1.0e+004, and 'Manual Baseline' is selected with a start cycle of 3 and an end cycle of 15. Both panels feature 'Analyze' and 'Help' buttons at the bottom.

- 3.4 Save the changes.

- 3.5 Select the wells containing samples and export the Report file **File>Export>Results...** as shown below:



- 3.6 To avoid confusion, save the file with the same name as used for the run file itself. Remember to save the file to an appropriate location.
- 3.7 When prompted, activate **Export only selected wells**, and click **OK**.
- 3.8 Open the saved .csv file with Excel or similar spreadsheet software.
- 3.9 Analyse each sample, starting with the controls, as shown in the flowchart below (details can also be found in the table shown beneath the flowchart) (The data is established using MicroAmp® Optical 8-strip PCR tubes. Use of different plastic consumables may void the data).



Sample	PNE MycAssay Ct	IAC MycAssay Ct	Interpretation
Negative Control	39.0 or Undetermined	Within 29.0-32.7	Negative Control acceptable
Negative Control	39.0 or Undetermined	<29.0 or >32.7	Failure in Negative Control
Negative Control	<39.0	Within 29.0-32.7	Contamination
Positive Control	Within 20.0-26.0	N/A	Positive Control acceptable
Positive Control	<20.0 or >26.0	N/A	Failure in Positive Control
Test Sample	39.0 or Undetermined	Within 29.0-32.7	Negative for <i>Pneumocystis</i>
Test Sample	<39.0	N/A	Positive for <i>Pneumocystis</i>
Test Sample	39.0 or Undetermined	<29.0 or >32.7	IAC failure in sample

4. Troubleshooting

4.1 The Negative Control has generated a positive signal in the FAM channel:

- Contamination occurred during the set up. Results from the entire run cannot be relied upon as accurate.
- Repeat the entire run taking great care when adding the templates, in particular, the Positive Control (Tube 4), to ensure that cross-contamination does not occur.
- Make sure that the work area and instruments are properly decontaminated before and after use.
- The Negative Control was incorrectly positioned in the instrument.
- Take care that the reaction tubes are placed in their designated sites and are annotated correctly within the software.

4.2 The Negative Control IAC Ct value is not within the acceptable range:

- The PCR has been inhibited.
- Ensure that the work area and instruments are thoroughly dry after the use of decontaminating agents prior to PCR set up.
- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.
- Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with unexpired kit if necessary.
- Either Tube 1 or 2 reagent was not added to the PCR reaction, or double the amount of Tube 2 was added.
- Repeat the run taking care in the set-up stage. Such errors can be detected by seeing higher or lower levels of liquid in one reaction tube compared to others.
- Non-recommended tubes or plates were used.
- Thresholds are only valid when using the recommended MicroAmp® tubes and caps.

4.3 The Positive Control is negative:

- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.
- Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with an unexpired kit if necessary.
- An error occurred during step 1.12 and the Positive Control template (Tube 4) was placed in the wrong reaction tube.
- Repeat the run, taking great care during the set-up stage. Such errors can be detected by seeing a higher level of liquid in one reaction, and a lower level in another, compared to normal.
- Either Tube 1 or 2 reagent was not added to the reaction.
- Repeat the run taking care in the set-up stage. Such errors can be detected by seeing lower levels of liquid in this reaction compared to others.
- The Positive Control was incorrectly positioned in the instrument.
- Take care that the reaction tubes are placed in their designated sites.

4.4 Test sample(s) are negative and the IAC is out of range:

- It is likely that the test sample(s) contain PCR inhibitors.
- Use a different DNA extraction method that removes inhibitors.
- Non-recommended tubes or plates were used.
- Thresholds are only valid when using the recommended MicroAmp® tubes and caps.

4.5 There are no results for any channel with any samples or controls:

- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.
- Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with an unexpired kit if necessary.

- The equipment used is not functioning optimally.
 - Please check that your Real-Time PCR instrument has an up-to-date service history and has been fully calibrated as described in its Installation and Maintenance Guide.
- An incorrect protocol file was used during the software set up.
 - Please refer to Section 2 and choose the correct Protocol file, as specified for each software type/version, from the Myconostica Protocol CD-ROM. Only the file appropriate to the software can be loaded. Repeat the run using the correct protocol file.

If you have further questions, or you experience any problems, please contact Technical Support (mycotech@myconostica.co.uk)

Performance Characteristics and Limitations

Analytical Sensitivity

Using the protocol described above, and a recombinant *Pneumocystis* DNA molecule generated at Myconostica, the Limit of Detection (LoD) for *Pneumocystis* was determined to be < 30 copies. This value was determined using a recombinant DNA plasmid harboring the target sequence. The *Pneumocystis* target sequence is mitochondrial, therefore, there will be numerous copies per cell, but it is not known how many.

Analytical Selectivity

Analytical selectivity was tested using DNA extracted from a variety of different fungal and non-fungal species. The following species did not report out a positive result; *Alternaria alternata*, *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *Blastomyces capitatus*, *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *Cladosporium spp.*, *Cryptococcus neoformans*, *Doratomyces microsporus*, *Fusarium solani*, *Histoplasma capsulatum*, *Rhizomucor pusillus*, *Rhodotomila rubra*, *Saccharomyces cerevisiae*, *Scedosporium apiospermum*, *S. prolificans*, *Sporothrix schenckii*, *Trichosporon capitatum*. The following bacterial species did not report a positive result; *Bordetella pertussis*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Haemophilus influenzae*, *Lactobacillus plantarum*, *Legionella pneumophila*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *S. pyogenes*, *S. salivarius*.

LICENSING

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